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“Immunohistochemical detection of EGFR, fibrillin-2, P-cadherin and AP2β as biomarkers for rhabdomyosarcoma diagnostics“

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Immunohistochemical detection of EGFR, fibrillin-2, P-cadherin and AP2β as biomarkers for rhabdomyosarcoma diagnostics

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Aims: Subclassification of rhabdomyosarcoma (RMS) has clinical relevance, as the two major subclasses embryonal (ERMS) and alveolar (ARMS) rhabdomyosarcoma differ greatly in terms of aggressiveness and prognosis. However, histological analysis is not always sufficient for an unequivocal subclassification of RMS. Furthermore, clinical presentation of ARMS has been reported to mimic other tumour types, specifically lymphoma. The aim was to determine the role of four biomarkers in the diagnosis of rhabdomyosarcoma.
Methods and results: Recently, we identified four potential biomarkers to subclassify RMS with high sensitivity and specificity. These included epidermal growth factor receptor (EGFR) and fibrillin-2 as markers for ERMS, and AP2β and P-cadherin as markers for translocation-positive ARMS. Here, we further validate the potential of these four markers in a second, independent patient cohort by immunohistochemistry on 80 sections of RMS biopsy specimens as well as a tissue microarray representing 18 different additional tumour types, including seven lymphomas. The combination of EGFR and fibrillin-2 was able to detect ERMS with a specificity of 76% and sensitivity of 90%. The combination of AP2β and P-cadherin detected ARMS with a specificity of 97% and sensitivity of 90%, data very similar to our previous study. Furthermore, all lymphomas were clearly negative for AP2β and P-cadherin.
Conclusions: These four biomarkers are suitable for clinical implementation in the future diagnosis of RMS.

Keywords: AP2β, diagnosis, EGFR, fibrillin-2, P-cadherin, rhabdomyosarcoma

Abbreviations: ARMS, alveolar rhabdomyosarcoma; DAB, diaminobenzidene; EGFR, epidermal growth factor receptor; ERMS, embryonal rhabdomyosarcoma; RMS, rhabdomyosarcoma; TMA, tissue microarray

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue tumour in childhood, with an overall incidence of 5–8% of childhood tumours. It falls into the class of small round blue cell tumors, is thought to derive from myogenic precursor cells and thus can occur at various locations in the body.

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Histologically, two main subtypes of rhabdomyosarcoma are distinguished with significant relevance as regards clinical outcome and survival. The more common embryonal rhabdomyosarcoma (ERMS) shows better response to established treatment regimens, reflected by an event-free 5-year survival rate of 70% compared with only 45% in children affected by alveolar rhabdomyosarcoma (ARMS).[1] This difference in treatment options and prognosis emphasizes the importance of a clear distinction between ERMS and ARMS upon diagnosing a child with RMS.

Unfortunately, histological discrimination between the different subtypes can sometimes be difficult. Furthermore, ARMS can present as widespread disease with an occult primary site mimicking other neoplasms such as leukaemia,[2,3] or lymphoma[4,5] rendering the diagnostic process even more challenging. Therefore, other methods have been designed to assist in the diagnosis of RMS. Apart from molecular methods such as polymerase chain reaction or fluorescence *in situ* hybridization to detect specific translocations in ARMS, the method of choice in the clinic is immunohistochemistry. Different myogenic marker proteins for RMS in general (desmin) or for RMS subgroups (myogenin) have been implemented in routine clinical diagnosis. Nevertheless, subgroup specificity of these markers leaves room for improvement.[6] Additional potential marker proteins for RMS subclassification have been detected in gene expression profiling studies of RMS.[7] Among them are epidermal growth factor receptor (EGFR) and fibrillin-2 as markers for ERMS and AP2β and P-cadherin as markers for translocation-positive ARMS. We have recently evaluated these four markers for their potential in aiding the subclassification of RMS tumours using a RMS tissue microarray (TMA) and found that they indeed allow immunohistochemical subclassification of RMS with high specificity and sensitivity.[8]

To validate these findings further for possible future clinical implementation on a second, independent patient cohort, we re-evaluated these markers on an additional 80 RMS tumour specimens. Furthermore, the suitability of the markers to distinguish ARMS from tumours with potential similar clinical appearance, especially lymphoma, was evaluated on a TMA representing 18 different non-RMS tumours.

Materials and methods

TUMOUR SPECIMENS

Single sections (2 μm) of 80 paraffin-embedded RMS specimens from patients diagnosed between 2001 and 2005 were obtained from the archives of the Department of Pathology at the University of Kiel, Germany. Histological classification was established locally and by referral to the Cooperative Weichteilsarkom Studiengruppe (CWS). All tumours were histologically subclassified as ARMS or ERMS.

Sections (2 μm) of a multi-tumour tissue array containing cores of 175 tumours from 18 different tissue origins and 38 cores from five different benign tissue types were obtained from the Institute of Surgical Pathology (University Hospital Zurich, Switzerland).

IMMUNOHISTOCHEMISTRY

Sections of both RMS tumours and the multi-tumour TMA were immunohistochemically stained using the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA) as described previously.[8] Briefly, slides were heated with either cell conditioner 1 (for EGFR, AP2β and P-cadherin) or cell conditioner 2 (fibrillin-2) and in some cases enzymatically predigested with Ventana protease 1 for 4 min (EGFR) or 8 min (fibrillin-2) to retrieve the antigens. The sections were then incubated for 32 min with primary antibodies against EGFR (clone 3C6, prediluted by Ventana) or P-cadherin (clone 56, 1:50; BD Transduction Labs, San Jose, CA, USA) or for 60 min with antibodies against AP2β (H-87, purified rabbit immunoglobulin fraction, 1:40; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or fibrillin-2 (polyclonal rabbit antibody, 1:200; Abcam plc, Cambridge, UK). Visualization of the antibodies was performed with either the Ventana iVIEW diaminobenzidene (DAB) detection kit (for EGFR, AP2β and P-cadherin) or the Ventana ultraVIEW DAB kit (for fibrillin-2). All tumour tissues were counterstained with haematoxylin.

EVALUATION OF IMMUNOHISTOCHEMICAL REACTIVITY

A scoring system based on three categories (0, 1 and 2) was used to grade immunoreactivity. In cases without any visible reactivity, the tumour was interpreted as negative and classified as 0. Cases with weak or moderate reactivity of a minority of cells were classified as 1. Tumours with reactivity of the majority of cells or heavy reactivity of a minority of cells were classified as 2. In order to rule out non specific background, reactivity was interpreted as specific only when found at the expected subcellular localization, i.e. at the cell periphery (EGFR and P-cadherin), in the nucleus (AP2β) or in the extracellular space (fibrillin-2). All sections were evaluated independently by two investigators.

Sensitivity and specificity of the antibody markers were calculated using the following formulae:

Sensitivity = number of true positives/(number of true positives + number of false negatives).

Specificity = number of true negatives/(number of true negatives + number of false positives).

Results

In a recent study, we investigated the potential of the four biomarkers EGFR, fibrillin-2, AP2β and P-cadherin for RMS subclassification by immunohistochemistry using a RMS TMA.[8] There, high specificity and sensitivity could be demonstrated, confirming earlier gene expression data. To validate these results on large single tumour sections, for this study we used sections of a set of 80 independent RMS tumours from 78 paediatric patients with a median age at diagnosis of 6.6 years.

According to histological classification, the study group was composed of 59 ERMS and 21 ARMS sections and included botryoid, pleomorphic and anaplastic variants in the ERMS group as well as the solid variant in the ARMS group (Table 1). All sections of the RMS tumours were immunohistochemically stained with antibodies against EGFR, AP2β, P-cadherin and fibrillin-2. The antibodies against the first three markers were the same as in our previous study, whereas the anti-fibrillin-2 antibody was now exchanged for a commercially available one. Immunoreactivity characteristics of the two anti-fibrillin-2 antibodies were very similar (data not shown). Representative reactivity of all four markers in both a typical ERMS and ARMS is shown in Figure 1. The frequency of immunopositivity among the RMS subgroups was determined and sensitivity and specificity were calculated (Table 2). Specific EGFR reactivity at the cell periphery was found in 55 of 59 ERMS sections and in 6 of 21 ARMS sections. Specific fibrillin-2 reactivity in the extracellular space was found in 56 of 59 ERMS sections, but in only 6 of 22 ARMS. Double positivity of these two markers was found in 53 of 59 ERMS but in only 5 of 21 ARMS, resulting in a sensitivity of 90% and specificity of 76% for the detection of ERMS.

Specific immunoreactivity of AP2β in the nucleus was detected in 19 of 20 ARMS sections and in 3 of 59 ERMS sections. Specific reactivity of P-cadherin at the cell periphery was found in 19 of 20 ARMS sections and in 8 of 59 ERMS. Double positivity for these two markers was found in 18 of 20 ARMS but only 2 of 59 ERMS, resulting in a sensitivity of 90% and specificity of 97% for the detection of ARMS with these two markers.

To evaluate the reproducibility of these results, we compared sensitivity and specificity determined in the present study with our previous TMA study investigating the same four markers on 252 tumour punches[8] and with a second TMA study testing only EGFR as a marker on 66 RMS tumours[9] (Table 3). This analysis revealed on the one hand that sensitivity of all markers on single sections was significantly higher than on TMAs. On the other hand, no major differences regarding specificity were found among the different studies.

The clinical presentation of ARMS can resemble other tumour types. Especially in cases with an occult primary tumour and widespread dissemination, ARMS can mimic leukaemia or lymphoma. In such cases an easy applicable diagnostic tool allowing discrimination from other tumours is desirable. We therefore tested the expression of our ARMS-specific markers AP2β and P-cadherin in a range of non-RMS tumours by immunohistochemistry of a TMA on which 18 different malignant and five benign tissue types were represented. As summarized in Table 4, specific AP2β reactivity was found in the majority of breast carcinomas (8/14) and melanomas (6/8). Furthermore, a minority of the cases of bladder (2/8), lung (2/12), ovarian (1/8), pancreatic (1/3) and skin (1/6) carcinomas were also AP2β positive. The remaining tumours, including lymphoma and different non-RMS sarcomas, were negative for AP2β. P-cadherin stained the majority of both tumours and benign tissues represented on the multi-tumour microarray, but not lymphomas, which were all clearly negative.

Table 1. Tumour characteristics

Rhabdomyosarcoma*	Patients (*n*)
ERMS	58
Botryoid	3/58
Pleomorphic	1/58
Anaplastic	4/58
Spindle cell	5/58
ARMS	20
Solid	7/20
Translocation positive†	8/20
Translocation negative	1/20

ERMS, embryonal rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma.

*All rhabdomyosarcoma tumours were immunopositive for desmin and myogenin, except two that were not tested.

†Translocation status was determined by specific polymerase chain reaction, but tested only in 11/20 ARMS. In addition, eight ERMS tumours were tested and found negative for *PAX/FKHR* translocations.

Discussion

Differences in treatment and prognosis among the different RMS subgroups as well as potential misdiagnosis of ARMS due to resemblance to other tumour types such as lymphoma underscore the importance of developing diagnostic tools for RMS (sub)classification. Gene expression profiling approaches carried out in recent years have allowed the detection of a range of

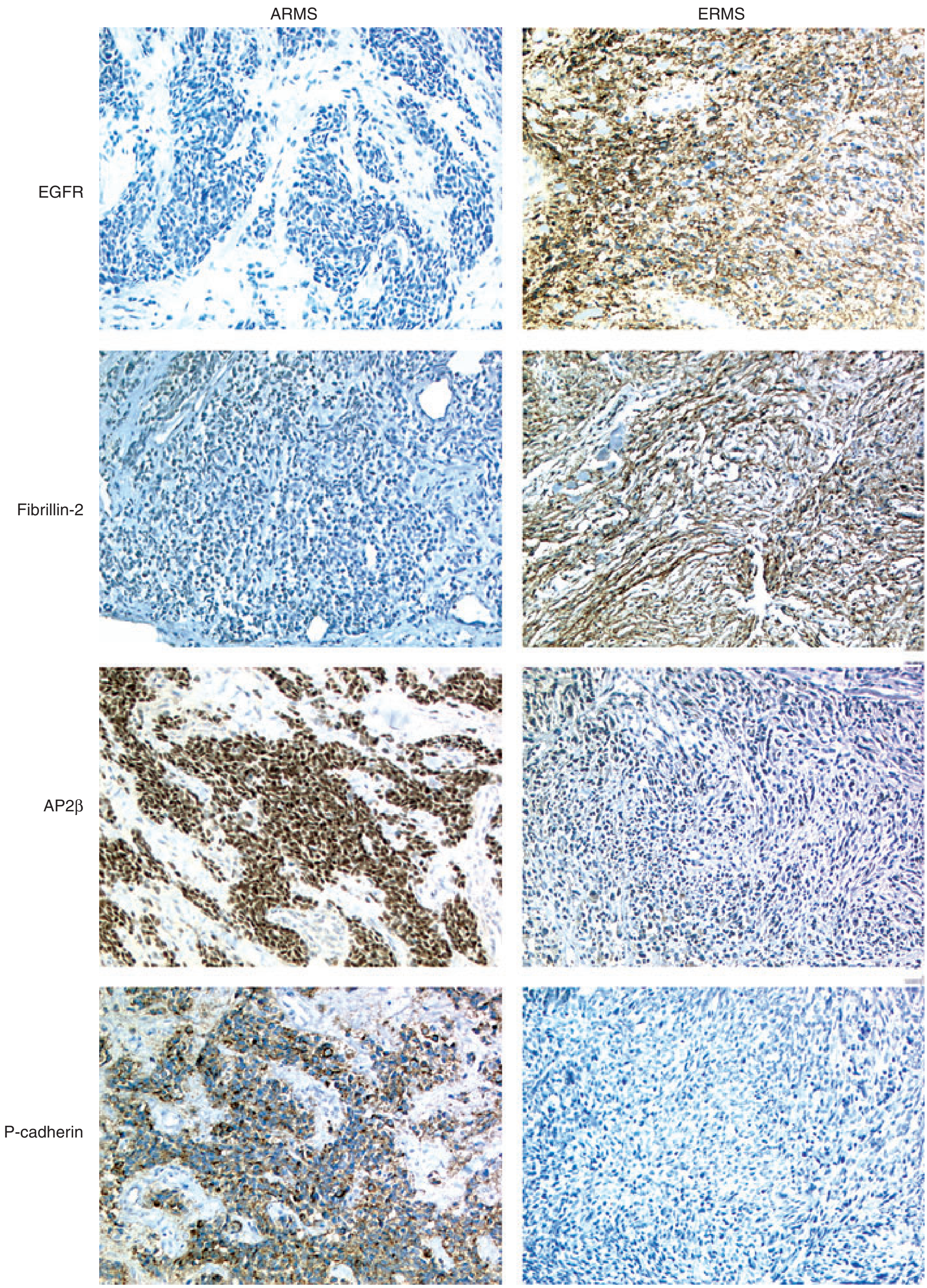

Figure 1. Immunoreactivity of a typical embryonal rhabdomyosarcoma (right panel) and alveolar rhabdomyosarcoma (left panel) with antibodies against AP2β, P-cadherin, epidermal growth factor receptor and fibrillin-2. Immunoreactivity was visualized with diaminobenzidene. For grading system, see Materials and methods.

Table 2. Summary of immunohistochemical data using four biomarkers

	ERMS*					ARMS*			
Antibody	Total	Anaplastic	Pleomorphic	Spindle cell	Botryoid	Total	Solid	Sensitivity (%)	Specificity (%)
EGFR	55/59	4/4	1/1	3/5	3/3	6/21	3/8	93.2	74.1
Fibrillin-2	56/59	4/4	1/1	4/5	3/3	5/21	2/8	94.9	76.1
EGFR + fibrillin-2	53/59	4/4	1/1	3/5	3/3	5/21	2/8	89.8	76.1
AP2β	3/59	0/4	0/1	0/5	0/3	19/20	7/7	95.0	94.9
P-cadherin	8/59	1/4	0/1	0/5	0/3	19/20	7/8	95.0	86.4
AP2β + P-cadherin	2/59	0/4	0/1	0/5	0/3	18/20	7/7	90.0	96.6

ERMS, embryonal rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma; EGFR, epidermal growth factor receptor.
*Two patients were represented by two sections.

Table 3. Comparative immunohistochemical data

Antibody	Study	ERMS	ARMS	Sensitivity (%)	Specificity (%)
EGFR	Ganti *et al.*[9]	26/34	5/32	76	84
	Wachtel *et al.*[8]	145/173	12/61	84	80
	Present study	55/59	6/21	93	74
Fibrillin-2	Wachtel *et al.*[8]	120/176	7/59	68	88
	Present study	56/59	5/21	95	76
EGFR + fibrillin-2	Wachtel *et al.*[8]	103/173	4/59	60	93
	Present study	53/59	5/21	90	76
AP2β	Wachtel *et al.*[8]	7/177	33/61	54	96
	Present study	3/59	19/20	95	95
P-cadherin	Wachtel *et al.*[8]	18/179	41/59	69	90
	Present study	8/59	19/20	95	86
AP2β + P-cadherin	Wachtel *et al.*[8]	0/176	32/59	54	100
	Present study	2/59	18/20	90	97

ERMS, embryonal rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma; EGFR, epidermal growth factor receptor.

potential biomarkers that might be suitable for RMS subclassification. Some of these biomarkers, i.e. EGFR and fibrillin-2 as ERMS-specific markers and AP2β and P-cadherin as ARMS-specific markers, have indeed been evaluated by immunohistochemistry on a RMS TMA representing several hundred tumour specimens.[8] The large number of samples that can be tested at once make such a TMA an ideal tool for initial screening of marker performance in terms of specificity and sensitivity. Nevertheless, for future implementation in a clinical setting, validation of the results with an independent patient cohort and on full tumour sections is indicated to exclude any potential bias due to the limited size of the tumour specimens on the TMA. Therefore, we validated the four markers on 80 single RMS tumour sections from 78 patients. Compared with

Table 4. Expression of ARMS-specific biomarkers in multiple tumour types

Tissue type		AP2β		P-cadherin	
Malignant	Total	Positive	Negative	Positive	Negative
Bladder	8	2	6	8	0
Brain	6	0	6	0	6
Breast	14	8	6	14	0
Colon	8	0	8	7	1
Kidney	10	0	10	5	5
Liver	5	0	5	3	2
Lung	12	2	10	12	0
Lymphoma	7	0	7	0	7
Melanoma	8	6	2	8	0
Ovary	8	1	7	8	0
Pancreas	3	1	2	4	0
Prostate	8	0	8	8	0
Sarcoma 2 Myxofibrosarcoma 2 Leiomyosarcoma 2 Synovial sarcoma 2 Undefined sarcoma	8	0	8	2	6
Seminoma	6	0	6	6	0
Skin	6	1	5	6	0
Teratoma	2	0	2	2	0
Thyroid	8	0	8	7	1
Uterus	7	0	7	7	0
Benign					
Kidney	4	0	4	4	0
Liver	8	1	7	8	0
Pancreas	2	0	2	2	0
Placenta	8	0	8	8	0
Tonsil	15	3	12	8	7

ARMS, alveolar rhabdomyosarcoma.

the tissue array data, sensitivity using full single sections was higher for all four markers. This result is not surprising, since in cases in which the tumour tissue is not uniformly positive, positive areas may not be represented on the TMA and staining may thus result in false-negative samples. On the other hand, no major difference in specificity between data from tissue array and from whole sections was detected, demonstrating that background reactivity of the antibodies was comparable. Furthermore, data from a third study, investigating EGFR expression in RMS subgroups, also produced similar results for this marker.[9]

The ARMS-specific markers AP2β and P-cadherin are expressed specifically in translocation-positive but not -negative ARMS.[8] However, the translocation status is not known for all tumours in this study, and this distinction was therefore not possible. Nevertheless, we found a high sensitivity of the two markers of 90% for the detection of ARMS. The remaining number of negative ARMS cases of about 10% is very similar to the number of true translocation-negative ARMS of 10–20% described in the literature.[10] This suggests that the sensitivity of our markers to detect translocation-positive ARMS is close to 100%. This is in line with the finding that these markers are present within the gene expression signature, which is a very specific and consistent indication for the presence of *PAX*-translocations in RMS,[7,11,12] suggesting that these genes are direct targets of the chimeric transcription factors *PAX3(or7)/FKHR.* Supporting evidence for this hypothesis has recently been obtained for AP2β.[13] Based on this high specificity of these markers for the detection of translocation-positive ARMS, it is reasonable to speculate that the two ERMS found in this study to be positive for both AP2β and P-cadherin also represent translocation-positive ARMS, even if there might be alternative translocations expressed.[7,10]

Due to its rapidly disseminating behaviour and the nature of its cellular morphology, namely small round blue cell tumours, ARMS can mimic the clinical presentation of lymphoma or leukaemia in cases where the primary tumour site is not detected. Testing 18 different non-RMS tumour types, we found that only breast carcinomas and melanomas were immunopositive for AP2β in significant numbers. AP2β alone therefore allows discrimination of ARMS from different tumours, including those with potentially similar appearance such as non-RMS sarcoma and lymphoma. Combination with markers specific for potentially similar tumours such as CD45 as a marker for lymphomas[14] could improve diagnosis further. On the other hand, many different tumour types on the array were positive for P-cadherin. This is not surprising, since most of the carcinomas are expected to express junctional proteins such as cadherins, taking their epithelial origin into account. More importantly, however, seven out of seven lymphomas were also negative for P-cadherin.

Taken together, our data suggest that the four markers are suitable to make the clinically relevant distinction between translocation-positive ARMS and ERMS and allow further discrimination of translocation-positive ARMS from other tumours, especially lymphoma. Therefore, these biomarkers represent important tools for RMS diagnosis.

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